NTP Research Concept: Alkylanilines

Project Leader:
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Nomination Background and Rationale:
2-Ethylaniline (2EA), 3-ethylaniline (3EA), and 3,5-dimethylaniline (3,5DMA) were nominated for toxicological characterization because of the potential for widespread human exposure, the limited availability of published toxicological data for this subclass of alkyl-substituted (ethyl-) anilines, and their structural similarities to the known animal carcinogens 2,6-dimethylaniline (2,6-DMA) and 2-methylaniline (2MA) which are known to form DNA adducts [1; 2] .

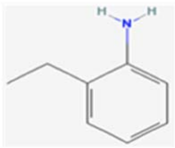
There is documented low level exposure of the general population to a large number of alkylanilines. Furthermore, there is strong evidence to suspect that many of the alkylanilines are metabolized to genotoxic species in humans. To better gauge the carcinogenic risk associated with exposure to the many alkylanilines we propose a broad class study that will address the relative genotoxic hazard posed by 14 members of this chemical class (Figure 1.).

<u>Exposure potential:</u>
A significant fraction of the alkylanilines suggested for study are used in the synthesis of dyes. In addition, a fraction of them are used as intermediates in the synthesis of pharmaceuticals, agrichemicals and photographic chemicals.

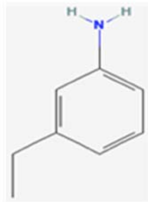
There is limited or no data on the production of most of the alkylanilines that are suggested for study. There are two exceptions, 2E6EA and 2M6EA, both involved in the synthesis of chloroacetanilide herbicides, had reported production volumes in 2006 of 10 to 50 million pounds.

Non-occupational exposure to arylamines (a subclass of which is the alkylanilines) has been well documented in cigarette smokers [3]. Smoking is associated with the increased rates of bladder cancer. The arylamines are believed to be the constituents of tobacco smoke that lead to the development of bladder cancer. According to hemoglobin adduct data the non-smoking population is exposed to a variety of alkylanilines including 2MA, 3MA, 4MA, 2,3DMA, 2,4DMA, 2,5DMA, 2,6DMA, 3,4DMA, 3,5DMA, 2EA, 3EA, and 4EA. Exposure to these alkylanilines potentially occurs through breathing ambient air containing combustion products or through the use of hair dyes. In non-smoking residents of Los Angeles detectable levels of 2EA, 3EA, and 3,5DMA (in addition to other alkylanilines suggested for study here) hemoglobin adducts were found, substantiating the claim that there is widespread exposure to these chemicals and that this exposure leads to detectable levels of macromolecular adducts. In the same study an association was found between 3,5DMA and 3EA hemoglobin adducts and bladder cancer risk in non-smokers, suggesting that exposure to alkylanilines leads to an increased cancer burden in the non-smoking population.

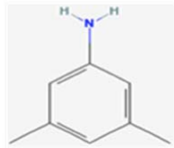
Concept Chemicals:



2-Ethylaniline

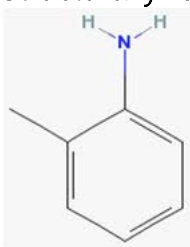


3-Ethylaniline

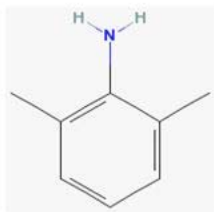


3,5-Dimethylaniline

Structurally related rodent carcinogens (NTP bioassay):

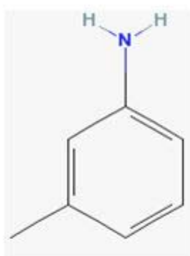


2-methylaniline

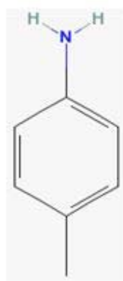


2,6-dimethylaniline

Structurally related alkylanilines with documented human exposure:

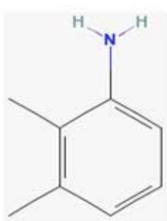


3-methylaniline

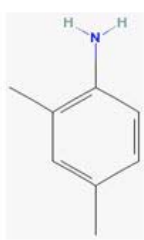


4-methylaniline

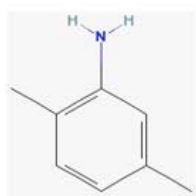
4-ethylaniline



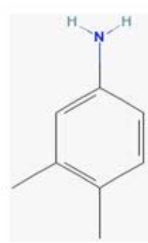
2,3-dimethylaniline



2,4-dimethylaniline



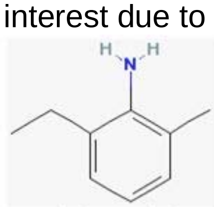
2,5-dimethylaniline



3,4-dimethylaniline

Other alkylanilines of interest due to high production volumes (>10 M lbs in 2006)

2,6-diethylaniline



2-methyl-6-ethylaniline

Figure 1. Structures of methyl- and ethyl-substituted anilines proposed for class study.

Another potential source of non-occupational exposure to chemicals in the alkylaniline class comes from both the biodegradation and hepatic metabolism of chloroacetaniliide herbicides [4; 5]. Specifically, alachlor is biodegraded to 2E6EA, whereas acetochlor and metolachlor are both biodegraded to 2M6EA. The environmental breakdown of these herbicides has the potential to contaminate the water supply.

Toxicology information**:**
As is the case with most anilines, high levels of the alkylanilines are anticipated to, or have been shown to, cause methemoglobinemia and interfere with blood oxygenation. Liver and kidney toxicity are also often observed following exposure to substituted anilines [6].

Although substituted anilines are generally negative in Ames studies they are considered genotoxic due to their propensity to form DNA adducts in *in vitro* and *in vivo* mammalian systems [1; 2; 7].

Key Issues:
The alkylanilines proposed for study are closely related structurally and would all likely be metabolized to N-hydroylamines in vivo [8]. A fraction of the alkylanilines may also be metabolically transformed to DNA reactive quinone imines [5]. Direct genotoxicity as a byproduct of N-hydroxylamine or quinone imine formation is the expected carcinogenic mode of action for the chemicals. Despite a common mode of action the alkylanilines will likely exhibit differential adduction potency related to the degree to which they form N-hydroxylamines and/or quinone imines and the relative stability of the penultimate reactive species. Ames mutagenicity failed in many cases to identify arylamines as mutagenic potentially due to the absence of cytosolic, phase II enzymes in the assay system. Furthermore, due to involvement of multiple metabolic steps (many of which are not mimicked with high integrity by cultured mammalian cells) and *in vivo* physiological states of the different organs that in influence the formation of the reactive species, characterization of DNA adduction potential should be done *in vivo*. By evaluating the DNA adduction potency of two alkylanilines that have already undergone testing in the carcinogenicity bioassay in parallel with the untested alkylanilines it will be possible to estimate the relative carcinogenic activity of the chemicals in this class.

Mutagenic activity of a chemical is function of both the capacity to adduct DNA and the capacity of the cell to respond to the damage. DNA adducts formed from distinct alkylanilines have already been shown to be differentially mutagenic [9]. Therefore, in addition to measuring levels of DNA adducts, it will be critical to gauge the *in vivo* mutagenic activity of the alkylanilines.

Genetic factors significantly influence both the formation of DNA reactive species from arylamines and the ultimate outcomes of exposure to this class of chemicals [10]. It is therefore critical to address the degree to which genetics influences the genotoxicity of the alkylanilines and to use this data to refine hazard assessments.

Proposed Approach:
The overall goal of this research project is to establish the relative genotoxicity of the different alkylanilines, to understand how genotoxicity translates to carcinogenicity and to characterize the effect genetic variation has on the genotoxicity of these chemicals. At the outset we plan to perform studies with all 14 of the chemicals that will address both *in vivo* DNA reactivity and mutagenicity. These studies will allow us to establish the relative genotoxic hazard of the 14 alkylanilines. A fraction of the alkylanilines will then be selected for study in a short-term carcinogenicity bioassay using transgenic mice. Chemicals will be selected for the carcinogenicity studies based on perceived carcinogenic hazard as determined by the initial genotoxicity studies. In parallel with the carcinogenicity studies we plan to evaluate, using cultured hepatocytes from a collection of genetically distinct mouse strains, the influence which genetics plays in determining the genotoxic potential of the alkylanilines. Included in the proposed studies will be two established rodent carcinogens which will be used to determine the relative genotoxicity and by extension carcinogenic potency of the untested chemicals. B6C3F1 mice will be used for the described studies because they are sensitive to the carcinogenic effects of 2-MA (NTP TR-153). We hypothesize that all the alkylanilines will differentially adduct DNA. One of the factors that will influence DNA reactivity and mutagenicity will include the position and size of alkyl substitutions. Previous studies suggest that substitution at the para position may facilitate formation of DNA reactive species [9]. Furthermore, it is hypothesized that the carcinogenic potency of the alkylanilines will be a function of both initial DNA reactivity and mutagenicity of the specific alkylaniline-DNA adducts. Such a hypothesis is in line with Lutz *et al*. who described the Carcinogen Binding Index which estimates the carcinogenic potential of a DNA reactive substance based on propensity to alkylate DNA in *in vivo* studies [11].

<u>Specific Aims</u>
Specific Aim 1: Characterize the relative genotoxic potency of the14 alkylanilines. These studies will compare a collection of alkylanilines (shown above) with respect to their capacity to produce DNA adducts, hemoglobin adducts, mutation of the Pig-A locus, erythrocyte micronuclei, and DNA fragmentation (Comet). These studies will be conducted using male B6C3F1 mice as they are likely to metabolize the alkylanilines to DNA reactive species [1]. Tissues collected for DNA adduct studies will include, but not be limited to bladder and liver. DNA adduction will be measured using accelerated mass spectrometry. Measurement of hemoglobin adducts will facilitate the translation of the studies to humans where alkylaniline exposure is determined by hemoglobin adducts [12].

Specific Aim 2: Test the carcinogenic activity of a subset of alkylanilines using Xpc-/+ p53-/+ transgenic mice. In order to determine if the studies described in specific aim 1 are predictive of carcinogenic activity we plan to study a subset of the alkylanilines in short-term cancer bioassays. Alkylanilines that are anticipated (based on tier 1 genotox studies) to be negative and those that are anticipated to be positive will be tested. Such an approach will allow us to gage the accuracy of the predictions made using the genotoxicity data. The Xpc-/+p53-/+ mice were selected for these studies because a closely related transgenic mouse strain (Xpa/p53+/-) were sensitive to arylamine carcinogenic activity in the liver and bladder as evidenced by the results of studies using P-cresidine and 2-aminoflourene [13]. Furthermore, unlike the Xpa-/+ p53-/+ mice, the Xpc-/+p53-/+ mice can be administered doses approximating those used in a traditional bioassay that employs B6C3F1 mice.

Specific Aim 3: Quantify the degree to which genetic variation influences the genotoxicity of a select set of alkylanilines. These studies will employ cultured hepatocytes from inbred strains of mice that exhibit genetic diversity at loci known to influence the genotoxicity of arylamines. Hepatocytes will be treated with equimolar doses of the select alkylanilines and DNA adduction will be measured**.**

Significance and Expected Outcomes:

The proposed research program will determine, relative to known rodent carcinogens, the genotoxic and mutagenic hazard of a collection of largely untested alkylanilines with widespread human exposure. Furthermore, it will provide information on the carcinogenic activity of this class of chemicals, the degree to which genetics influences the genotoxicity of these chemicals and, potentially, the specific genetic determinants of variable susceptibility to the genotoxic action of this subclass of arylamines.

References:

[1] P.L. Skipper, L.J. Trudel, T.W. Kensler, J.D. Groopman, P.A. Egner, R.G. Liberman, G.N. Wogan, and S.R. Tannenbaum, DNA adduct formation by 2,6-dimethyl-, 3,5-dimethyl-, and 3-ethylaniline in vivo in mice. Chem Res Toxicol 19 (2006) 1086-90.

[2] J.D. Duan, A.M. Jeffrey, and G.M. Williams, Assessment of the medicines lidocaine, prilocaine, and their metabolites, 2,6-dimethylaniline and 2-methylaniline, for DNA adduct formation in rat tissues. Drug Metab Dispos 36 (2008) 1470-5.

[3] M.S. Bryant, P. Vineis, P.L. Skipper, and S.R. Tannenbaum, Haemoglobin adducts of aromatic amines in people exposed to cigarette smoke. IARC Sci Publ (1988) 133-6.

[4] D.M. Stamper, and O.H. Tuovinen, Biodegradation of the acetanilide herbicides alachlor, metolachlor, and propachlor. Crit Rev Microbiol 24 (1998) 1-22.

[5] S. Coleman, R. Linderman, E. Hodgson, and R.L. Rose, Comparative metabolism of chloroacetamide herbicides and selected metabolites in human and rat liver microsomes. Environ Health Perspect 108 (2000) 1151-7.

[6] Y. Sakuratani, S. Sato, S. Nishikawa, J. Yamada, A. Maekawa, and M. Hayashi, Category analysis of the substituted anilines studied in a 28-day repeat-dose toxicity test conducted on rats: Correlation between toxicity and chemical structure, Taylor & Francis, 2008, pp. 681 - 696.

[7] M.E. Kugler-Steigmeier, U. Friederich, U. Graf, W.K. Lutz, P. Maier, and C. Schlatter, Genotoxicity of aniline derivatives in various short-term tests. Mutat Res 211 (1989) 279-89.

[8] J.W. Gorrod, and D. Manson, The metabolism of aromatic amines. Xenobiotica 16 (1986) 933-55.

[9] M.M. Marques, L.L. Mourato, M.T. Amorim, M.A. Santos, W.B. Melchior, Jr., and F.A. Beland, Effect of substitution site upon the oxidation potentials of

alkylanilines, the mutagenicities of N-hydroxyalkylanilines, and the conformations of alkylaniline-DNA adducts. Chem Res Toxicol 10 (1997) 1266-74.
[10] D.W. Hein, M.A. Doll, A.J. Fretland, M.A. Leff, S.J. Webb, G.H. Xiao, U.S. Devanaboyina, N.A. Nangju, and Y. Feng, Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. Cancer Epidemiol Biomarkers Prev 9 (2000) 29-42.
[11] W.K. Lutz, In vivo covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. Mutation Research/Reviews in Genetic Toxicology 65 (1979) 289-356.
[12] J. Gan, P.L. Skipper, M. Gago-Dominguez, K. Arakawa, R.K. Ross, M.C. Yu, and S.R. Tannenbaum, Alkylaniline-hemoglobin adducts and risk of non-smoking-related bladder cancer. J Natl Cancer Inst 96 (2004) 1425-31.
[13] C.F. van Kreijl, P.A. McAnulty, R.B. Beems, A. Vynckier, H. van Steeg, R. Fransson-Steen, C.L. Alden, R. Forster, J.W. van der Laan, and J. Vandenberghe, Xpa and Xpa/p53+/- knockout mice: overview of available data. Toxicol Pathol 29 Suppl (2001) 117-27.